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Limited tolerance towards folded elements during secretion of the autotransporter Hbp

Wouter S. P. Jong,¹

Corinne M. ten Hagen-Jongman,¹

Tanneke den Blaauwen,² Dirk Jan Slotboom,³

Jeremy R. H. Tame,⁴ David Wickström,⁵

Jan-Willem de Gier,⁵ Ben R. Otto¹ and Joen Luirink^{1*}

¹Department of Molecular Microbiology, Institute of Molecular Cell Biology, Vrije Universiteit, 1081 HV Amsterdam, the Netherlands.

²Department of Molecular Cytology, Swammerdam Institute for Life Sciences, University of Amsterdam, 1098 SM Amsterdam, the Netherlands.

³Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, the Netherlands.

⁴Protein Design Laboratory, Yokohama City University, Yokohama 230-0045, Japan.

⁵Department of Biochemistry and Biophysics, Arrhenius Laboratories, Stockholm University SE-106 91 Stockholm, Sweden.

Summary

Many virulence factors secreted by pathogenic Gram-negative bacteria belong to the autotransporter (AT) family. ATs consist of a passenger domain, which is the actual secreted moiety, and a β -domain that facilitates the transfer of the passenger domain across the outer membrane. Here, we analysed folding and translocation of the AT passenger, using *Escherichia coli* haemoglobin protease (Hbp) as a model protein. Dual cysteine mutagenesis, instigated by the unique crystal structure of the Hbp passenger, resulted in intramolecular disulphide bond formation dependent on the periplasmic enzyme DsbA. A small loop tied off by a disulphide bond did not interfere with secretion of Hbp. In contrast, a bond between different domains of the Hbp passenger completely blocked secretion resulting in degradation by the periplasmic protease DegP. In the absence of DegP, a translocation intermediate accumulated in the outer membrane. A similar jammed intermediate was formed upon insertion of a calmodulin folding moiety into Hbp. The data suggest that Hbp can fold in the periplasm but must

retain a certain degree of flexibility and/or modest width to allow translocation across the outer membrane.

Introduction

Despite their complex cell envelope, Gram-negative bacteria secrete a wide variety of proteins into the extracellular milieu (Nandakumar *et al.*, 2006). Several pathways have evolved to enable protein transfer across the inner membrane, the periplasm and the outer membrane. The autotransporter (AT) pathway, also known as type Va secretion, appears the most widely distributed secretion system and is typically used by virulence factors with diverse roles in pathogenesis. The AT dogma is deceptively simple in the sense that the AT, as implied by its name, carries all information for translocation across the periplasm and outer membrane within the protein itself (Henderson *et al.*, 2004).

Autotransporters are synthesized as large precursor proteins that contain three domains: (i) the signal sequence at the N-terminus to allow targeting to and initiation of transfer across the Sec-translocon, a protein conducting channel in the inner membrane used by most secreted and membrane proteins, (ii) the secreted passenger domain that carries the actual effector function of the AT and (iii) the β -domain at the C-terminus that adopts a β -barrel conformation in the outer membrane and plays a crucial role in transfer of the passenger domain across the outer membrane (Henderson *et al.*, 2004; Jacob-Dubuisson *et al.*, 2004). The mechanism of secretion of ATs is unclear and controversial at the molecular level.

The most debated issues relate to the existence of a periplasmic intermediate in the translocation process and the nature and oligomeric organization of the translocator pore in the outer membrane. Considerable evidence suggests that ATs temporarily reside in the periplasm (Jose *et al.*, 1996; Brandon and Goldberg, 2001; Veiga *et al.*, 2004; Skillman *et al.*, 2005; Rutherford *et al.*, 2006). However, the extent of folding in this compartment and the tolerance of the translocator towards passage of folded structures is unclear and may vary between different ATs. Folding of the passenger in the periplasm has implications for the nature and dimensions of the translocator pore. Initially, it was proposed that the pore is formed by a single C-terminal β -domain that transfers the passenger in an

Accepted 9 January, 2007. *For correspondence. E-mail joen.luirink@falw.vu.nl; Tel. (+31) 0 20 5987175; Fax (+31) 0 20 5986979.

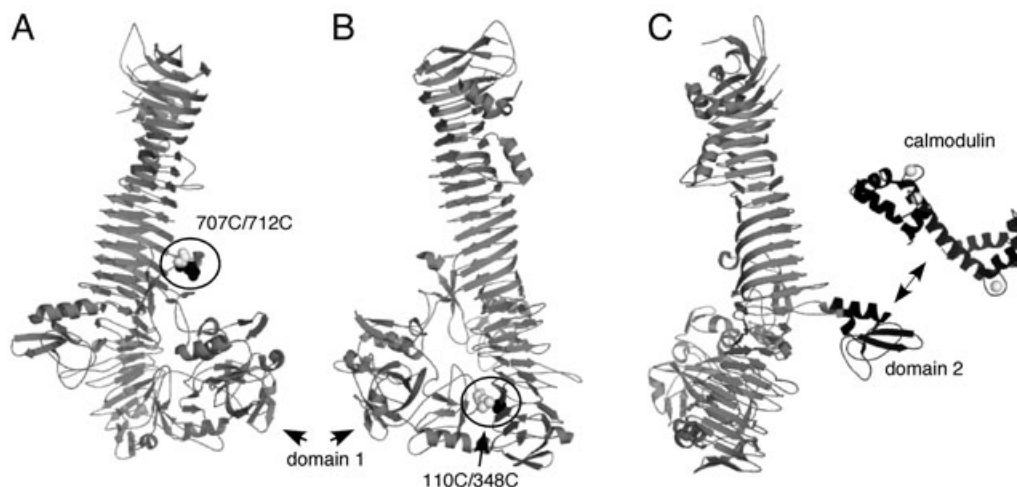


Fig. 1. Hbp derivatives used in this study. Hbp residues targeted for mutation are indicated in the crystal structure of the secreted Hbp passenger [1WXR (PDB); Otto *et al.*, 2005].

A. Hbp707C/D712C: Ala⁷⁰⁷ (white spheres)/Asp⁷¹² (black spheres) → Cys⁷⁰⁷/Cys⁷¹².

B. Hbp110C/348C: Leu¹¹⁰ (white spheres)/Gly³⁴⁸ (black spheres) → Cys¹¹⁰/Cys³⁴⁸.

C. Hbp(Calm): Hbp domain 2 (aa 483–555; black) substituted by the Ca²⁺-loaded form of human calmodulin [1CLL (PDB); Chattopadhyaya *et al.*, 1992]. Ca²⁺ ions in calmodulin are represented by white spheres. The structure of Hbp has been rotated with respect to the vertical axis to provide the best view of the three mutants. Images were created using MacPyMOL. A colour version of this figure is available online (Fig. S1).

extended, unfolded conformation (Klauser *et al.*, 1992). Indeed, the recent crystal structure of the β -domain of NalP reveals a narrow (10×12 Å) 12-stranded β -barrel pore that precludes passage of folded structures more complex than an α -helix (Oomen *et al.*, 2004). On the other hand, purified β -domain of the IgA protease was shown to form multimeric ring-like structures that may enclose a channel with a larger diameter (20 Å), possibly compatible with partially folded passenger structures (Veiga *et al.*, 2002; Veiga *et al.*, 2004). An alternative pore model involves Omp85 that is considered a general insertase for outer membrane proteins. Omp85 might directly act as a channel for the passenger or indirectly modulate the conformation and dimensions of a β -barrel translocator (Voulhoux *et al.*, 2003; Oomen *et al.*, 2004).

Haemoglobin protease (Hbp) is a key virulence factor that is produced and secreted in large amounts via the AT mechanism by a pathogenic *Escherichia coli* strain during peritonitis (Otto *et al.*, 1998; Otto *et al.*, 2002). Hbp degrades haemoglobin and subsequently binds the released haem as an easily accessible source of iron. Hbp belongs to the SPATE family (serine protease ATs of *Enterobacteriaceae*) that possess a consensus serine protease motif. This motif is present in many important secreted virulence factors from *E. coli* and *Shigella* species (Henderson and Nataro, 2001).

Data with respect to AT folding and outer membrane translocation presented thus far have been obtained before detailed molecular models of any AT passenger domain had been reported (Klauser *et al.*, 1990; 1992;

Suzuki *et al.*, 1995; Jose *et al.*, 1996; Maurer *et al.*, 1997; Konieczny *et al.*, 2000; Lattemann *et al.*, 2000; Fischer *et al.*, 2001; Veiga *et al.*, 2004; Jose and Zangen, 2005; Skillman *et al.*, 2005; Rutherford *et al.*, 2006). We have recently determined the crystal structure of Hbp (110 kDa), the first structure of a SPATE and the first complete structure of a secreted AT passenger domain (Fig. 1; Otto *et al.*, 2005). Therefore, we considered it opportune to re-examine folding and translocation of ATs, using Hbp as a model substrate allowing structure-based mutants with minimal perturbation of the native passenger structure. Using paired cysteine mutagenesis we obtained evidence for considerable folding of the Hbp passenger in the periplasm. 'Locking' the periplasmic intermediates by disulphide bridging was incompatible with secretion only when the cysteines were distant in the primary structure. In this case, a DegP-sensitive secretion intermediate was formed in the outer membrane that appeared partly exposed at the cell surface. A similar jammed intermediate was formed when a small, separate, Hbp domain was replaced by a small folded calmodulin moiety. The data suggest that Hbp can fold in the periplasm but must retain a certain degree of flexibility and/or modest width to allow translocation across the outer membrane.

Results

Rationale for the selection of paired cysteine residues

The crystal structure of the secreted Hbp passenger shows a long right-handed β -helical stem, slightly kinked

in the centre where a small β -stranded domain of unknown function (domain 2) protrudes from the stem. In addition, a large N-terminal globular domain (domain 1) is present that contains the serine protease site (Fig. 1; Otto *et al.*, 2005). To obtain insight into the folding state of the passenger domain during transit in the periplasm we have introduced paired cysteine residues in the Hbp passenger that does not contain any cysteines of its own. Positions were chosen that appear juxtaposed in the crystal structure and, aided by the program SSBOND (Hazes and Dijkstra, 1988), were predicted to be optimal for disulphide bridge formation. One pair is also close in primary structure (707C/712C) and located in a loop that extrudes from the main β -helix stem just above the catalytic domain 1 (Fig. 1A). The second pair is remote in primary structure (110C/348C) but close in tertiary structure. Leu¹¹⁰ and Gly³⁴⁸ participate in a long and narrow contact region that connects the catalytic domain to the lower part of the cylindrical β -stem (Fig. 1B).

Expression and secretion of paired cysteine mutants

The paired cysteine mutants were cloned under *lac* promoter control and expressed in the wild-type *E. coli* strain DHB4. Cells were grown in minimal medium to early log phase before induction of Hbp expression with isopropyl β -D-thiogalactopyranoside (IPTG) for 2 h. Samples were then withdrawn and centrifuged to separate cells and medium. To monitor expression and secretion of Hbp, both fractions were analysed by SDS-PAGE and Coomassie staining (Fig. 2A).

Under these conditions, wild-type Hbp is efficiently expressed, processed and secreted as shown by the appearance of the 110 kDa product in cells (lane 2) and medium (lane 3), as compared with the empty vector control (lane 1). The 110 kDa product represents the Hbp passenger domain (Otto *et al.*, 1998; van Dooren *et al.*, 2001; Sijbrandi *et al.*, 2003), which was confirmed by immunoblotting (data not shown). The passenger form in the cell fraction is accessible to protease added to intact cells and hence translocated and exposed at the cell surface (data not shown). As expected, a ~28 kDa product accumulated in the cells (Fig. 2A, lane 2), corresponding to the cleaved Hbp β -domain (van Dooren *et al.*, 2001). The identity of the Hbp β -domain was confirmed by immunoblotting (data not shown). The paired cysteine mutant Hbp110C/348C showed only trace amounts of Hbp both in the cell and supernatant fraction indicating poor expression (lanes 4 and 5). In contrast, expression, processing and secretion of Hbp707C/712C was indistinguishable from wild-type Hbp indicating that transfer of this paired cysteine mutant across the cell envelope proceeds unperturbed (lanes 6 and 7).

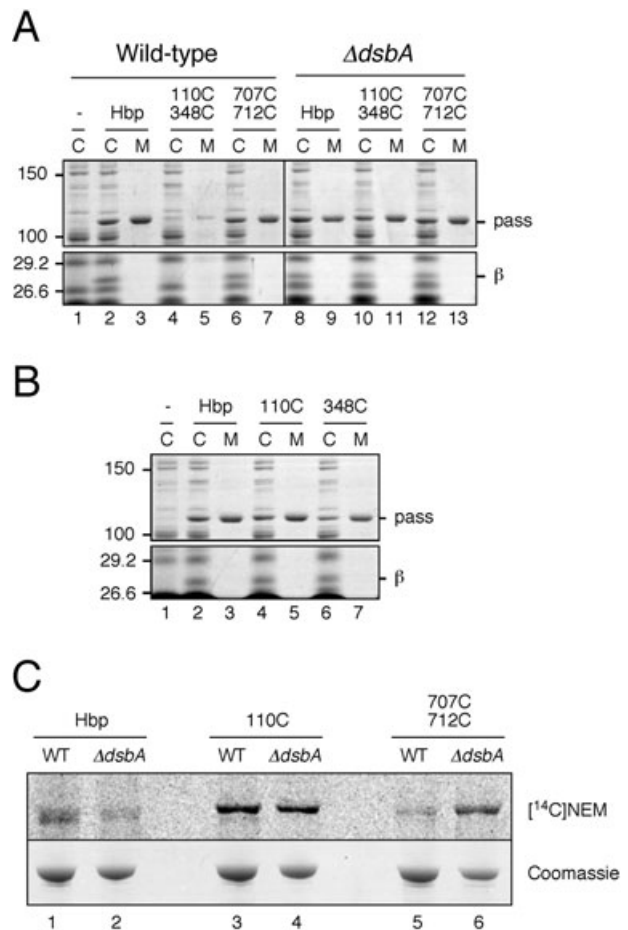


Fig. 2. Disulphide bonding and secretion of Hbp cysteine mutants. **A.** Expression and secretion of wild-type Hbp and the paired cysteine mutants (Hbp110C/348C and Hbp707C/712C) in the *dsbA* strain DHB4 (lanes 8–13) and its isogenic wild-type DHB4 (lanes 2–7). Cells harbouring the constructs cloned into the expression vector pEH3 or empty vector (lane 1) from overnight cultures were resuspended in fresh medium and their growth was continued. When cultures reached early log phase, expression of Hbp(mutants) was induced with 1 mM of IPTG. Samples were collected 2 h after induction, separated in cells (C) and spent medium (M) and analysed by SDS-PAGE and Coomassie staining. The relevant areas of the same gel are shown. Molecular mass (kDa) markers are indicated at the left side of the panels. Hbp passenger (pass) and β -domain (β) are indicated at the right hand side of the panels. **B.** Expression and secretion of wild-type Hbp and single cysteine mutants Hbp110C and Hbp348C in strain DHB4 analysed as described in A. **C.** Analysis of medium fractions derived from cultures of DHB4 (lanes 1, 3, 5) or DHBA (2, 4, 6) expressing wild-type Hbp, Hbp110C or Hbp707C/712C collected 90 min after induction of expression. Half of each fraction was treated with [¹⁴C]NEM and analysed by SDS-PAGE and phosphorimaging (top), whereas the other half was left untreated and analysed by SDS-PAGE and Coomassie staining (bottom). See *Experimental procedures* for more details.

The poor expression of the Hbp110C/348C might be related to structural effects of the mutations *per se* or to the formation of intra or intermolecular disulphide-bonded species in the oxidizing milieu of the periplasm (Ritz and Beckwith, 2001). Conceivably, these forms are incompatible with translocation across the outer membrane and prone to degradation in the periplasm (Ruiz and Silhavy, 2005). To investigate this issue in more detail, the single cysteine mutants Hbp110C and Hbp348C were analysed separately for expression and secretion. As shown in Fig. 2B, both mutants behaved like wild-type Hbp, strongly suggesting that the diminished expression of the paired Hbp110C/348C mutant is due to intramolecular disulphide bond formation. To test this possibility, Hbp110C/348C was expressed in a strain that lacks the major periplasmic oxidoreductase, DsbA, and is hence unable to form disulphide bonds in the periplasm (DeLisa *et al.*, 2003). Clearly, expression and secretion are restored to wild-type levels in this strain (Fig. 2A, lanes 10 and 11). Consistently, partial restoration of Hbp110C/348C secretion was also achieved in a wild-type strain by the addition of the reducing agent β -mercaptoethanol during expression (data not shown). Together, the results imply that Hbp folds sufficiently well in the periplasm to undergo disulphide bonding between domains that are close in tertiary but remote in primary structure. Apparently, the 'locked' Hbp110C/348C intermediate is unable to pass the outer membrane and, consequently, almost completely degraded.

Unlike Hbp110C/348C, the double cysteine mutant Hbp707C/712C was secreted efficiently in both the wild-type and the *dsbA*⁻ strain. This indicated that either the presence of a disulphide bond did not affect secretion or, alternatively, that a disulphide bond could not be formed at all. To verify that a disulphide bond could be formed in the secreted Hbp707C/712C, the mutant Hbp was isolated from the culture medium, digested with trypsin and the resulting peptides were analysed by tandem mass spectrometry. The presence of a trypsin cleavage site between the two cysteines (K709/D710) allowed us to demonstrate unambiguously that secreted Hbp707C/712C passenger contains a disulphide bond (Fig. S2).

Most likely, disulphide bonding in Hbp707C/712C has taken place in the periplasm prior to translocation across the outer membrane. To confirm this, we assessed the accessibility of the Hbp707C/712C to derivatization with [¹⁴C]N-ethyl-maleimide (NEM), a reagent that specifically and covalently binds to free thiol groups of unbonded cysteine side-chains. Hbp707C/712C and control constructs were expressed for 1.5 h in a wild-type and *dsbA*⁻ strain in minimal medium lacking methionine and cysteine to minimize extracellular oxidoreductase activity (Brandon and Goldberg, 2001). Culture medium samples were collected, directly incubated with [¹⁴C]NEM and analysed by

SDS-PAGE followed by phosphorimaging to visualize derivatization (Fig. 2C, upper panel) and by Coomassie staining to estimate secreted Hbp levels (Fig. 2C, lower panel). Only minor [¹⁴C]NEM labelling was detected when Hbp707C/712C was expressed in wild-type cells (lane 5), similar to the background labelling of wild-type Hbp that is devoid of cysteines (lane 1). However, Hbp707C/712C labelling was significantly increased upon expression in a *dsbA*⁻ background whereas labelling of wild-type Hbp remained unchanged (lane 6 versus 2). As a further control on the specificity and efficiency of [¹⁴C]NEM labelling, the single cysteine mutant Hbp110C was strongly labelled irrespective of the presence or absence of DsbA in the host strain (lanes 3 and 4). Together, our results clearly demonstrate that DsbA catalyses intramolecular disulphide bond formation in Hbp707C/712C in the periplasm. Importantly, this covalently modified intermediate is still translocation competent and efficiently secreted into the culture medium.

In conclusion, the data suggest that the Hbp passenger folds in the periplasm to a considerable extent. However, some degree of flexibility appears critical as covalent linkage of folded domains completely blocks translocation across the outer membrane and results in degradation of the trapped intermediate. In contrast, a small loop tied off by a disulphide bond seems fully compatible with efficient transfer across the outer membrane.

Expression and secretion of Hbp fused to calmodulin

The paired cysteine mutants described above suggest a correlation between the size and flexibility of folded Hbp intermediates and the competence for translocation across the outer membrane. To address this supposition with a method independent of disulphide bonding, we decided to introduce a well-characterized cysteineless folding domain into the Hbp passenger. To minimize perturbation of the basic β -stem structure and domain architecture of the native passenger, we chose to replace domain 2, a small (75 amino acids) separate domain of unknown function that protrudes from the β -stem just above the kink (Fig. 1C). As heterologous folding domain we selected calmodulin (145 amino acids) the folding stability of which is strongly dependent on the presence of Ca²⁺ ions. The Ca²⁺-loaded calmodulin forms an exceptionally stable dumbbell structure (Fig. 1C) whereas the apo-form is significantly unfolded at normal temperatures. This feature allows for control of the folding state of calmodulin by manipulating the Ca²⁺ level (Guerini and Krebs, 1983; Zhang *et al.*, 1995; Adams *et al.*, 2005). To ensure optimal conformational flexibility with respect to the β -stem, calmodulin was flanked by Pro-Gly-Gly spacers. The resulting construct was named Hbp(Calm). As a control, we created Hbp(Δ dom2) that lacks domain 2 entirely.

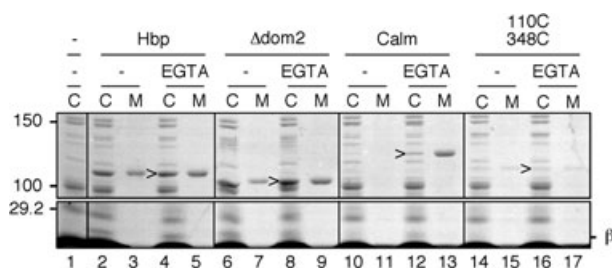


Fig. 3. EGTA-dependent secretion of Hbp fused to calmodulin. Expression and secretion of wild-type Hbp, Hbp(Δ dom2), Hbp(Calm) and Hbp110C/348C in strain DHB4, analysed as described in Fig. 2A except that EGTA (200 μ M) was added to half of the cultures concomitant with IPTG. Data are displayed as in Fig. 2A, except that the (mutant) Hbp passengers are indicated with '>'.

To investigate the effect of folding on the secretion competence of the chimera, Hbp(Calm) and the control constructs [wild-type Hbp and Hbp(Δ dom2)] were expressed in the wild-type DHB4 strain in minimal medium containing 100 μ M of CaCl_2 to induce folding of the calmodulin domain. Alternatively, growth was performed in the presence of 200 μ M ethylene glycol tetraacetic acid (EGTA) to chelate Ca^{2+} and thus to elicit a more flexible conformation of the calmodulin domain. Expression and secretion of Hbp(-derivatives) was monitored as before by analysing cell and medium fractions using SDS-PAGE and Coomassie staining (Fig. 3).

First, we verified that domain 2 is not required for expression and secretion of Hbp. Upon growth and induction, the passenger of Hbp(Δ dom2) could be observed in both the cell and medium fraction irrespective of the presence or absence of Ca^{2+} in the culture medium (lanes 6–9), similar to wild-type Hbp (lanes 2–5). Also, accumulation of cleaved β -domain was found under these conditions. We conclude therefore that domain 2 is dispensable for efficient expression, processing and secretion of Hbp.

We then analysed secretion of Hbp(Calm) under the same conditions. Upon growth and induction in the presence of Ca^{2+} , neither Hbp(Calm) passenger nor β -domain could be detected in either the cell or medium fractions (lanes 10 and 11). In light of the results obtained with the Hbp110C/348C mutant, we deduced that this poor expression is likely due to degradation of a folded and trapped intermediate. Consistently, the ~122 kDa Hbp(Calm) passenger was specifically secreted upon growth in the presence of EGTA (lane 13), a condition that is expected to prohibit stable folding of the calmodulin moiety. Furthermore, the cleaved β -domain could be identified in the cell fraction (lane 12) as confirmed by immunoblotting (data not shown). Immunoblot analysis further showed that growth in the presence of EGTA did not evoke leakage of periplasmic factors into the culture medium, arguing against a general permeabilization of the outer

membrane under these conditions (data not shown). To exclude any other non-specific effects of EGTA that might influence secretion of trapped Hbp intermediates, we verified that expression and secretion of the cysteine mutant Hbp110C/348C were not restored by EGTA (lanes 14–17).

Collectively, the data suggest that the extent of folding in the periplasm is a critical parameter for the translocation competence of Hbp. Secretion of Hbp is rather tolerant with respect to the attachment of flexible domains to the β -stem structure. In contrast, even small domains that are solidly folded block translocation of Hbp, leading to degradation.

Degradation of secretion incompetent Hbp mutants by the periplasmic protease DegP

The data presented in Figs 2 and 3 suggest that Hbp derivatives that have a propensity to form folded and inflexible structures are prone to degradation. Conceivably, these intermediates initially accumulate in the periplasm in a secretion incompatible, folded, form. It has been shown previously that the expression of heterologous or misfolded proteins in the periplasm elicits a cell envelope stress response that is characterized by upregulation of the periplasmic protease DegP (Ruiz and Silhavy, 2005). To examine cell envelope stress upon expression of our Hbp constructs in the wild-type DHB4 strain, we monitored the steady state levels of DegP by immunoblotting of cell fractions (Fig. 4A). Expression of wild-type Hbp and Hbp707C/712C only slightly induced DegP expression (lanes 1, 2 and 4). In contrast, cells that express Hbp110C/348C or Hbp(Calm) show markedly increased DegP levels indicative of severe cell envelope stress (lanes 3 and 5).

This finding suggested that DegP is directly involved in degradation of the folded, translocation incompetent Hbp110C/348C and Hbp(Calm) intermediates in the periplasm. To examine this possibility, expression of the Hbp derivatives was determined in a *degP*⁻ strain (Fig. 4B). Clearly, both Hbp110C/348C and Hbp(Calm) were expressed and accumulated in the cells as ~142 kDa (lane 14) and ~152 kDa (lane 18) products respectively, corresponding to the expected molecular mass of the unprocessed (pro-)form of the derivatives. Indeed, the presence of both passenger and β -domain in the accumulated products was confirmed by immunoblotting (data not shown). Evidently, a prolonged presence of the pro-form of Hbp110C/348C and Hbp(Calm) in the periplasm does not result in significant restoration of secretion (lanes 15 and 19) arguing that these intermediates are intrinsically translocation incompetent. As expected, the absence of DegP did not significantly change expression and secretion of wild-type Hbp and Hbp707C/712C (lanes 2, 3, 6, 7, 12, 13, 16, 17).

To examine the connection between processing, secretion and degradation of Hbp in more detail, we

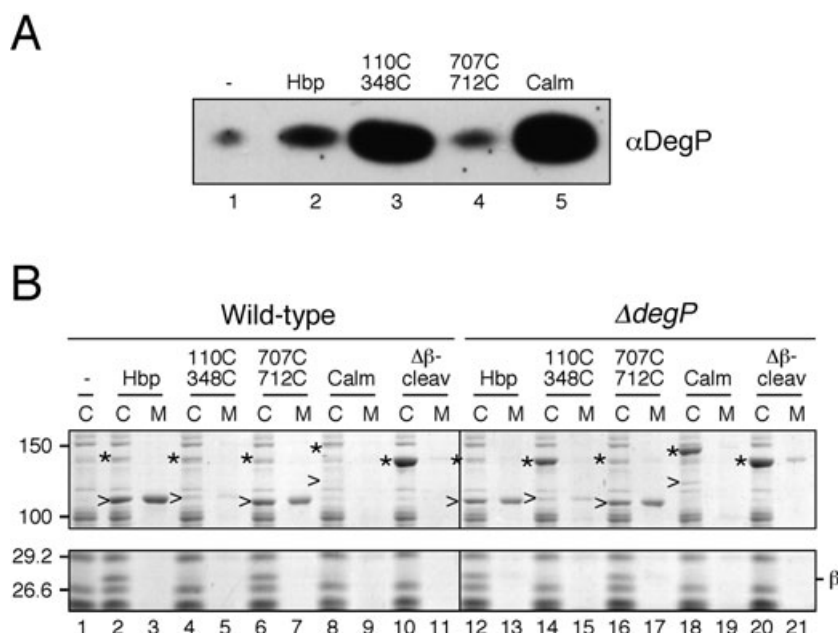


Fig. 4. Degradation of secretion incompetent Hbp mutants by DegP.

A. DegP levels in DHB4 cells expressing Hbp, Hbp110C/348C, Hbp707C/712C or Hbp(Calm). Cells were grown and induced for Hbp expression as in Fig. 2A. Cell fractions were analysed by SDS-PAGE and immunoblotting using an antiserum against DegP.

B. Analysis of expression and secretion of Hbp, Hbp110C/348C and Hbp707C/712C, Hbp(Calm) and Hbp(Δβ-cleav) in the *degP*⁻ strain KS476 (lanes 12–21) and wild-type strain DHB4 (lanes 2–11) as in Fig. 2A. Results are displayed as in Fig. 2A, except that the (mutant) Hbp passengers are indicated with '>' and the pro-forms of (mutant) Hbp are indicated with '*'.

constructed an Hbp mutant that we expected to be translocated across the outer membrane in an unprocessed, cell-associated form (see under *Secretion incompetent Hbp derivatives are stuck in the outer membrane*). In this mutant, Hbp(Δβ-cleav), the cleavage site between the passenger and β-domain, has been compromised. Expression of Hbp(Δβ-cleav) results in accumulation of uncleaved (pro-)Hbp in the cell fraction, as confirmed by immunoblotting (data not shown), irrespective of the presence of DegP in the host strain (lanes 10 and 20). Together with the localization of Hbp(Δβ-cleav) at the cell surface (see under *Secretion incompetent Hbp derivatives are stuck in the outer membrane*), this suggests that it is the localization of an Hbp secretion intermediate rather than its processing *per se* that determines the sensitivity towards degradation by DegP.

Together, the data demonstrate that DegP plays a pivotal, perhaps exclusive role in the degradation of intermediates of Hbp that fail to be translocated across the outer membrane.

Secretion incompetent Hbp derivatives are stuck in the outer membrane

As shown above, the Hbp folding mutants Hbp110C/348C and Hbp(Calm) accumulate in unprocessed (pro-)form in *degP*⁻ cells. Conceivably, these mutants initiate translocation of the passenger unit across the outer membrane until the inflexible domain is reached that jams the translocator and prevents further transport.

To investigate whether this is a valid scenario, the outer membrane localization of Hbp110C/348C and Hbp(Calm)

in the *degP*⁻ strain was examined. Cells were lysed in a French pressure cell and centrifuged to separate soluble from insoluble (membrane associated or aggregated) material. The insoluble fraction was subjected to isopycnic sucrose gradient centrifugation (Fig. 5). Analysis of the fractions by immunoblotting revealed that both mutants are predominantly found in the dense outer membrane fractions. For reasons that are unclear, some contamination of the outer membrane fractions with inner membranes was observed upon expression of Hbp(Calm) as apparent from the presence of the inner membrane protein Leader peptidase (Lep).

To corroborate the outer membrane localization of Hbp110C/348C and Hbp(Calm), we used an alternative strategy. Cells were lysed and the total membranes were collected by centrifugation. This fraction was treated with sodium lauryl sarcosinate (Sarkosyl) to extract the inner membranes. Using this procedure, both mutants were almost exclusively detected in the insoluble outer membrane fraction (Fig. S3). Together, the fractionation experiments indicate that both Hbp110C/348C and Hbp(Calm) accumulate in unprocessed form in the outer membrane.

If these mutants are genuine translocation intermediates that are jammed during passage of the outer membrane, it is expected that parts of the proteins are surface exposed. To investigate this possibility, the localization of the Hbp derivatives was analysed by immunofluorescence microscopy upon expression in *degP*⁻ cells. Cells were grown in minimal medium, induced for Hbp expression, fixed, and subjected to indirect immunofluorescence using a polyclonal antiserum directed against the Hbp passenger domain (Fig. 6). Under these conditions, cells

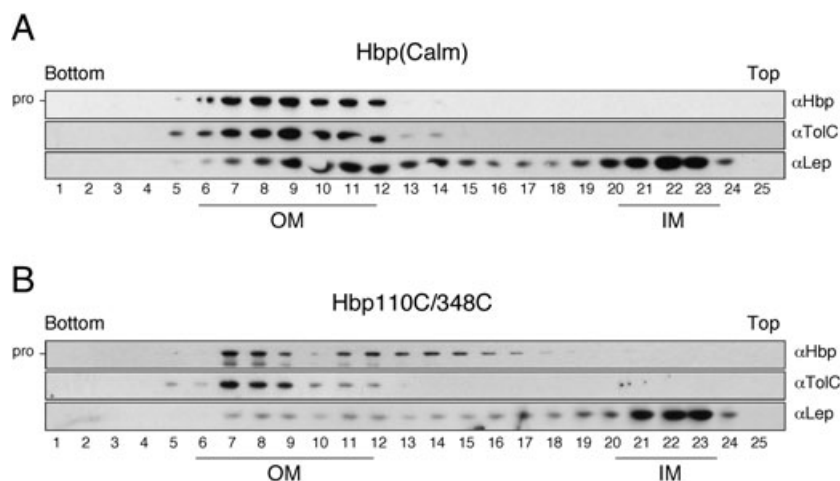


Fig. 5. Secretion incompetent Hbp derivatives are localized in the outer membrane. Membranes of KS476 cells expressing (A) Hbp(Calm) or (B) Hbp110C/348C were separated by sucrose gradient centrifugation. Fractions of equal volume from the gradients were analysed by SDS-PAGE and immunoblotting using antiserum against Hbp (top panel). To verify the membrane separation, both the outer membrane protein TolC (centre panel) and inner membrane protein Leader peptidase (Lep) (bottom panel) were detected in the fractions by immunoblotting. The pro-form (pro) of the Hbp derivatives is indicated.

are expected to remain intact and impermeable towards antibodies allowing selective labelling of surface-exposed Hbp. To verify maintenance of outer membrane integrity during the labelling procedure, a translocation incompetent mutant, Hbp(Pass), was constructed that lacks the C-terminal β -domain and accumulates in the periplasmic space of *degP*⁻ cells (data not shown). Significant labelling of Hbp(Pass) was not observed unless cells were first permeabilized upon treatment with ethylene diamine tetraacetic acid (EDTA) and lysozyme (Fig. 6L versus Fig. 6N). As a positive control we used Hbp($\Delta\beta$ -cleav) that we expected to be translocated but not to be released from the cells, based on data described above under *Degradation of secretion incompetent Hbp mutants by the periplasmic protease DegP*. Indeed, clear circumferential labelling of the cells was observed indicating surface exposure (Fig. 6J), as opposed to control cells that carry an empty vector and showed no labelling (Fig. 6B).

Expression of wild-type Hbp resulted in slightly weaker circumferential labelling of the cells (Fig. 6D). This is consistent with previous suggestions that part of the Hbp passenger remains bound or rebinds to the cell surface after translocation across the outer membrane (Otto *et al.*, 1998; van Dooren *et al.*, 2001). Expression of Hbp110C/348C resulted in strong circumferential labelling (Fig. 6F) arguing that part of the passenger domain is surface exposed. Surface presentation was also observed for Hbp(Calm) although the pattern was rather punctuated (Fig. 6H). Perhaps, translocation of Hbp(Calm) is confined to specific areas of the outer membrane, a supposition that needs more testing. Importantly, the immunofluorescence data strongly suggest that the passenger domains of both Hbp110C/348C and Hbp(Calm) are (at least partly) exposed at the cell surface.

In conclusion, the localization experiments indicate that the secretion incompetent mutants Hbp110C/348C and Hbp(Calm) accumulate in the outer membrane in an

unprocessed form with part of their passenger exposed at the cell surface and part exposed in the periplasm (accessible to DegP). Most likely and excitingly, these forms therefore represent genuine translocation intermediates that got jammed during translocation across the outer membrane.

Jammed translocation intermediates remain translocation competent

Assuming that Hbp110C/348C and Hbp(Calm) form translocation intermediates in the outer membrane, we wondered if, in principle, these intermediates were still translocation competent. To address this issue, we investigated by pulse-chase analysis whether jammed Hbp(Calm) intermediates in the outer membrane could be translocated upon relaxation of the calmodulin folding state by chelating Ca^{2+} with EGTA.

Hbp(Calm) was expressed in *degP*⁻ cells for 40 min and pulse-labelled for 5 min with [³⁵S]methionine. Then, cells were chased with cold methionine and EGTA was added to half of the cultures whereas the other half was left untreated. Samples were taken at indicated time points and centrifuged to separate cell and medium fractions that were analysed by SDS-PAGE and phosphorimaging (Fig. 7). Clearly, pro-Hbp(Calm) accumulated in the pulse-labelled cells whereas also some cleaved passenger was detected (lane 1). The identity of the bands was confirmed by immunoprecipitation using anti-Hbp antibodies (data not shown). Most of the accumulated Hbp(Calm) remained stable in unprocessed form over time irrespective of the presence of EGTA in the growth medium (lanes 2–9). Strikingly, a small but significant and gradually increasing amount of passenger domain was detected in the medium fraction during chase in the presence of EGTA (lanes 15–18). In contrast, only trace amounts of passenger were detected in the medium of untreated cells

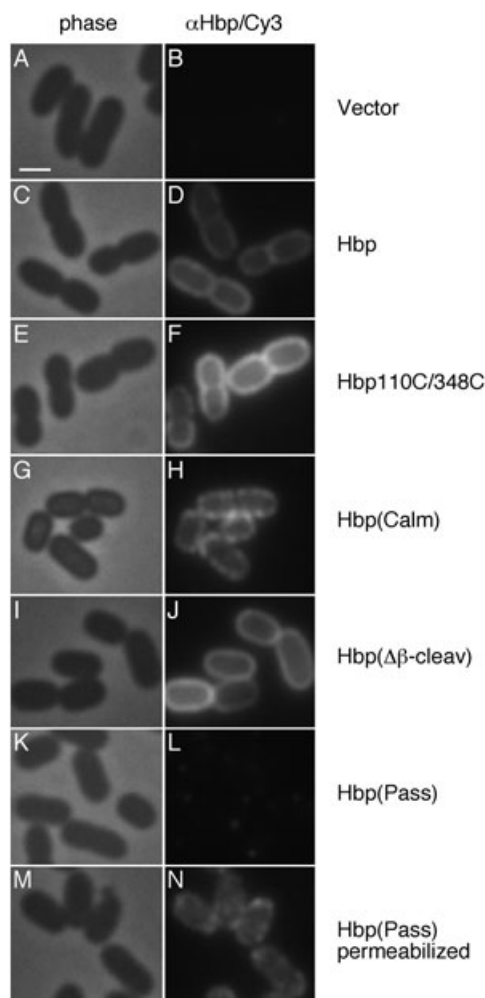


Fig. 6. Surface presentation of Hbp translocation intermediates in *degP*⁻ cells. KS476 cells expressing Hbp (derivatives) or carrying an empty vector (pEH3) were collected 1 h after induction with 200 μ M IPTG and subjected to indirect immunofluorescence using a polyclonal antiserum against the Hbp passenger and a Cy3-labelled conjugate (B, D, F, H, J, L, N). In addition, the corresponding fields are shown by phase microscopy (A, C, E, G, I, K, M). In the case of Hbp(Pass), half of the cells was permeabilized prior to labelling, as indicated. An exposure time of 0.033 s was used to obtain fluorescent images, except for Hbp(Pass) images where 0.1 s was used. The scale bar is 1 μ m.

(lanes 10–14). Addition of EGTA did not evoke the appearance of proteins other than Hbp in the medium (lanes 10–18 and data not shown) arguing against aspecific permeabilization of the outer membrane by EGTA. To further confirm the integrity of the outer membrane under the experimental conditions, we repeated the pulse chase procedure using an Hbp construct that lacks the C-terminal β -domain. This construct accumulates as passenger in the periplasm of *degP*⁻ cells (Fig. 6 and data not shown) and was retained in the cells even in the presence of EGTA (data not shown).

Collectively, the data suggest that a fraction of the accumulated pro-Hbp(Calm) intermediate has regained trans-

location competence in the presence of EGTA by adopting a more flexible apo-conformation of the calmodulin moiety allowing secretion and processing of the chimera.

Discussion

Gram-negative bacteria have an amazing capacity to secrete large amounts of sizeable molecules via the AT pathway (Henderson *et al.*, 2004). Despite its apparent simplicity, many fundamental questions concerning the mechanism of AT secretion still remain. Does the AT passenger domain temporarily reside and fold in the periplasm prior to translocation across the outer membrane? What is the nature and size of the translocator in the outer membrane? Is it the AT β -domain in a monomeric or multimeric conformation, or is an external translocator involved such as the recently identified Omp85 insertase (Voulhoux *et al.*, 2003)? Is translocation of the passenger vectorial, from C- to N-terminus? How is translocation energized? Many studies have addressed the mechanism of autotransport by replacing part of the passenger domain by heterologous proteins that are known to fold and form disulphide bonds in the periplasm. However, secretion of the chimeric ATs, sometimes expressed in heterologous host organisms, was reported to be variable, leading to incongruent models for AT folding and translocation (Klauser *et al.*, 1990; 1992; Suzuki *et al.*, 1995; Jose *et al.*, 1996; Maurer *et al.*, 1997; Konieczny *et al.*, 2000; Lattemann *et al.*, 2000; Fischer *et al.*, 2001; Veiga *et al.*, 2004; Jose and Zangen, 2005; Skillman *et al.*, 2005; Rutherford *et al.*, 2006). Therefore, we considered it opportune to re-examine this topic making use of the crystal structure of the native *E. coli* AT Hbp (110 kDa) that we recently determined, the first complete structure of a secreted AT passenger domain (Fig. 1; Otto

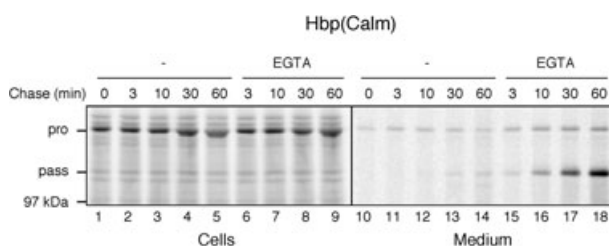


Fig. 7. Rescue of jammed Hbp(Calm). Pulse-chase analysis to monitor secretion of initially jammed Hbp(Calm) intermediates upon addition of EGTA. KS476 cells harbouring pEH3-Hbp(Calm) were induced for 40 min, radiolabelled with [³⁵S]methionine for 5 min and chased with cold methionine for 1 min. Then, EGTA (1 mM) was added to half of the cells (lanes 6–9 and 15–18), whereas the other half was left untreated (lanes 1–5 and 10–14). Samples were taken at indicated time points, separated in cells and spent medium and analysed directly by SDS-PAGE and phosphorimaging (cell fractions; lanes 1–9) or after TCA precipitation (medium fractions; lanes 10–18). The pro-form (pro) and passenger (pass) of Hbp(Calm) is indicated.

et al., 2005). Our data provide evidence that the Hbp passenger domain temporarily resides in the periplasm where considerable folding takes place prior to translocation. Yet, the intermediate has to retain a certain degree of flexibility to prevent congestion of the translocation apparatus and degradation by the periplasmic protease DegP.

The overall structure of the Hbp passenger shows a long right-handed β -helical stem with small loops and two major domains protruding from the stem (Fig. 1; Otto *et al.*, 2005). When we introduced cysteines at positions 707 and 712 of the Hbp passenger a disulphide bond was formed intracellularly, tying off a small loop that projects from the β -helical stem. The observation that formation of this bond did not interfere with efficient secretion of the passenger unambiguously shows that the Hbp translocator to some extent tolerates the passage of folded structures. Interestingly, the recently elucidated crystal structure of the β -domain of NalP (Oomen *et al.*, 2004), which is highly homologous to the Hbp β -domain, shows a rather narrow (10–12 Å), pore-like conformation consisting of 12 transmembrane strands. Efficient secretion of the Hbp707C/712C passenger and cysteine containing AT passengers (Brandon and Goldberg, 2001; Skillman *et al.*, 2005; Letley *et al.*, 2006) is difficult to reconcile with translocation through this narrow monomeric β -domain via the so-called hairpin mechanism. In this model, the C-terminal linker region inserts into the adjacent β -domain as a transient hairpin structure. The passenger domain is then pulled through the translocator from the C- towards the N-terminus initiated by rapid folding of the autochaperone unit that is located adjacent to the linker region (Oliver *et al.*, 2003). For the Hbp707C/712C passenger, this would imply that at some point in translocation the space occupied in the translocator is at least equal to the total diameter of three bundled polypeptide chains. This seems incompatible with the internal diameter of the β -domain of NalP which would only allow for the simultaneous presence of two polypeptide chains (Oomen *et al.*, 2004), implying that a larger pore exists during translocation of the Hbp passenger. In this respect, it should be mentioned that flexibility and species variation in internal diameter of AT β -domains cannot be excluded. Notably, the Hia translocator domain which is trimeric but also composed of 12 transmembrane β -strands (four strands from each subunit) and has the same external diameter as NalP, was shown to accommodate three α -helices (one from each subunit) (Meng *et al.*, 2006).

Although some secondary structure of the Hbp passenger may be permitted during secretion, the tolerance of the translocator towards folded elements is limited. Hbp(Calm), in which domain 2 was replaced by calmodulin that folds into a rigid dumbbell-shaped structure in the presence of Ca^{2+} (Guerini and Krebs, 1983; Zhang *et al.*,

1995), was shown to accumulate in the outer membrane where it is partly exposed at the cell surface and vulnerable to the periplasmic protease DegP suggesting it becomes obstructed during translocation. This observation is in conflict with two recent studies in which fusions of the ATs EspP and IgA protease to CtxB and scFv- V_{HH} -fragments respectively, were reported to translocate efficiently despite evidence for folding of the fusion partners in the periplasm (Veiga *et al.*, 2004; Skillman *et al.*, 2005). The reason for the apparent discrepancy is unclear but may relate to the smaller dimensions of CtxB and scFv- V_{HH} -fragments [$\sim 27 \times 35 \times 38$ Å and $\sim 28 \times 30 \times 40$ Å/ $24 \times 26 \times 43$ Å respectively (Desmyter *et al.*, 2002; Merritt *et al.*, 2002; Zahnd *et al.*, 2004)] compared with calmodulin [$\sim 45 \times 45 \times 65$ Å (Chattopadhyaya *et al.*, 1992)]. Also, these reporter domains may fold into a more relaxed conformation than Ca^{2+} -loaded calmodulin. Alternatively, particular characteristics of individual ATs may play a role. For instance, the β -domain of IgA protease has been reported to form a multimeric ring-shaped complex (Veiga *et al.*, 2002) that might accommodate larger folded structures. It is also relevant to note that most of the passenger domain has been deleted in the EspP and IgA protease constructs whereas the basic structure of Hbp(Calm) is still intact which might preserve the natural kinetics of biogenesis (see below).

The secretion incompatibility of large folded structures was corroborated by analysis of a paired cysteine mutant (Hbp110C/348C), in which cysteines are positioned at the interface between the β -stem and the catalytic domain (Fig. 1B). Secretion of this Hbp derivative was almost completely blocked unless the host cells lacked the oxidoreductase DsbA. Similar to Hbp(Calm), the disulphide-bonded Hbp110C/348C was efficiently degraded by DegP. Only in a *degP*⁻ background significant accumulation of unprocessed Hbp was observed in the outer membrane with at least part of the molecule exposed to the cell surface as evident from immunofluorescence studies. These data support a model in which Hbp transiently resides in the periplasm where it is accessible to chaperones and folding catalysts that assist folding into a protease-resistant structure. Folding of this large molecule may be time-consuming and require temporary tethering of the pro-form to the inner membrane by an unusual signal peptide that is used by Hbp and several other ATs to pass the inner membrane (Sijbrandi *et al.*, 2003; Szabady *et al.*, 2005). Interestingly, the folding intermediate is still translocation competent unless the catalytic domain is covalently linked to the β -stem domain. Apparently, the domains must be extricable or bendable in order to be accommodated by the flexible and/or spacious translocator. Possibly, the catalytic domain hinges downwards prior to or during translocation to be positioned in line with the β -stem and reduce the maximal diameter of

the translocated molecule. At this point, it is important to note that translocation of such folded passenger structures is compatible neither with a monomeric β -domain pore (Skillman *et al.*, 2005) nor with the IgA type multimeric β -domain structure (Veiga *et al.*, 2002) even when individual domains can move without restraint. Rather, translocation would require an external translocator of considerable size [Omp85? (Voulhoux *et al.*, 2003)].

Alternatively, it is possible that the folding step is followed by unfolding prior to or during translocation followed by refolding at the cell surface. This seemingly complicated and energetically unfavourable mechanism may be required to ensure protection of pro-Hbp against proteases in the periplasm and at the same time allow translocation through a narrow outer membrane pore. In both scenarios, the disulphide bond in Hbp110C/348C or the calmodulin domain in Hbp(Calm) is expected to obstruct the translocation channel and prevent further transport. More paired cysteine mutants are required to assess the extent of folding in the periplasm. It is a formal possibility that different folding states are sampled in the periplasm resulting in disulphide bond formation between cysteines irrespective of their position. However, the efficiency of the disulphide bond formation in Hbp110C/348C, together with data obtained with other paired cysteine mutants (data not shown) suggest that disulphide bond formation is a valuable marker to monitor folding of the Hbp passenger and its consequences for translocation.

In support of the above model, it seems likely that in the periplasm calmodulin does fold in the absence of Ca^{2+} , but into a more relaxed conformation (Guerini and Krebs, 1983; Zhang *et al.*, 1995) that is still compatible with translocation. Interestingly, the Ca^{2+} -dependent obstruction of the intermediate is in principle reversible. Chelating the Ca^{2+} from a jammed translocation intermediate in the outer membrane partly rescues secretion of the chimeric Hbp indicating that Hbp domains in the periplasm can remain translocation competent for prolonged times.

In view of the size and complexity of the Hbp molecule, it is conceivable that general periplasmic chaperones assist in folding and/or protection against proteolysis. Although our study does not address this issue directly, the chaperone/folding catalyst DsbA and the chaperone/protease DegP are not required for efficient expression and secretion of wild-type Hbp. In contrast, DegP appeared indispensable for surface expression of the *Shigella flexneri* AT lcsA (Purdy *et al.*, 2002). Notably, DegP does play a pivotal role in the quality control of Hbp, efficiently degrading mutant Hbp derivatives that are hampered in their translocation across the outer membrane.

Excitingly, Hbp110C/348C and Hbp(Calm) accumulate in the outer membrane with a surface-exposed domain

and a periplasmic domain arguing that they represent passenger domains that are 'caught in the act of translocation'. Although the exact topology of the intermediates remains to be determined, it seems most likely that the C-terminus of the passenger is surface exposed in support of the hairpin model. In any case, the intermediates provide a unique opportunity to determine the structure and composition of the translocator in action.

Experimental procedures

Strains and media

Strains DHB4, DHBA (*dsbA::Km*) and KS476 (*degP::Km*) are *E. coli* MC1000 derivatives and have been described previously (Strauch and Beckwith, 1988; DeLisa *et al.*, 2003). Unless stated otherwise, cells were grown at 30°C in M9 medium (Miller, 1992) containing 0.1% casaminoacids (Difco, Detroit, MI, USA) and 0.2% glucose. Overnight cultures were grown with 0.4% glucose. When appropriate, streptomycin (30 $\mu\text{g ml}^{-1}$), kanamycin (30 $\mu\text{g ml}^{-1}$) and chloramphenicol (20 $\mu\text{g ml}^{-1}$) were added to the medium.

Reagents, enzymes and sera

Restriction enzymes, T4-DNA ligase and Lumi-Light Western blotting substrate were purchased from Roche Applied Science. Pfu-Ultra DNA Polymerase was obtained from Stratagene, [^{35}S]Methionine from Amersham Biosciences and [^{14}C]NEM from PerkinElmer. All other chemicals were supplied by Sigma-Aldrich. Antisera against Hbp(J40), OmpA and Lep were from our own collection. The antisera against TolC and DegP were kind gifts from V. Koronakis (University of Cambridge, Cambridge, UK) and J. Beckwith (Harvard Medical School, Boston, USA) respectively. The Cy3-conjugated donkey anti-rabbit antibody was from Jackson ImmunoResearch.

Plasmid construction

Hbp and its derivatives were expressed from vector pEH3 (Hashemzadeh-Bonehi *et al.*, 1998). Details on the construction of plasmids used in this study can be found online as *Supplementary material*.

Derivatization with [^{14}C]NEM

Cells from overnight cultures were washed with M9 medium containing a cysteine- and methionine-free amino acid mix and 0.2% glucose, and subsequently resuspended and grown in the same medium. When cultures reached early log-phase, expression of Hbp(-derivatives) was induced with 1 mM IPTG. Spent medium samples were collected 90 min after induction. Of each sample, one half was directly subjected to derivatization with [^{14}C]NEM whereas the other half was left untreated. For derivatization, 1 ml of medium was treated with 27 μM [^{14}C]NEM and incubated at 30°C for 30 min. After addition of 500 μl of quench buffer (Tris-HCl,

pH 7.5/150 mM NaCl/10 mM NEM), the mixture was incubated at 30°C for another 5 min. Samples (treated and untreated) were subjected to tricarboxylic acid (TCA) precipitation and analysed by 4–12% NuPAGE/MOPS (Invitrogen).

Membrane separation by sucrose gradient centrifugation

Cells were induced with 200 µM of IPTG when cultures reached an OD₆₆₀ of ~ 0.3 and were harvested 1 h after induction. Membranes were isolated from ~300 OD₆₆₀ units of cell material and separated into inner and outer membranes by isopycnic sucrose gradient centrifugation essentially as described (De Vrije *et al.*, 1987), with the following modifications: dithiothreitol and EDTA were omitted from all buffers and phenyl methane sulphonyl fluoride was substituted by Complete EDTA-free protease inhibitor (Roche Applied Science). Furthermore, sucrose gradients were centrifugated for 20 h at 25 000 rpm using a TFT41.14 rotor (Kontron Instruments). Fractions withdrawn from the gradients were analysed by 11% SDS-PAGE and immunoblotting.

Indirect immunofluorescence microscopy

All procedures were carried out as described (Den Blaauwen *et al.*, 2003) except that for non-permeabilized cells treatment with Triton X-100, EDTA and lysozyme were omitted. Furthermore, cells were incubated with a polyclonal primary antibody against the Hbp passenger and a Cy3-conjugated donkey anti-rabbit secondary antibody.

Pulse-chase analysis

Cells from overnight cultures were resuspended in fresh medium and grown to an OD₆₆₀ of 0.4. Then, cells were washed and resuspended in M9-medium containing a cysteine- and methionine-free amino acid mix and 0.2% glucose. After recovery for 15 min, cells were induced with 1 mM IPTG for 40 min, subsequently labelled with 10 µCi ml⁻¹ [³⁵S]methionine for 5 min and chased with 2 mM cold methionine. After 1 min, EGTA (1 mM) was added to half of the cells whereas the other half was left untreated. Samples were taken at indicated time points and immediately added to an equal volume of ice-cold M9 medium to stop the chase. Samples were then separated in cells and spent medium by centrifugation at 4°C for 5 min at 8000 g and analysed directly by 9% SDS-PAGE and phosphorimaging (cells) or after TCA precipitation (medium fractions).

Sample analysis

SDS-PAGE gels were stained with Coomassie brilliant blue G (Janssen Chimica). Radiolabelled proteins were visualized by phosphorimaging using a Molecular Dynamics PhosphorImager 473. Gels containing [¹⁴C]NEM derivatized proteins were immersed in Amplify solution (Amersham) prior to phosphorimaging.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Hbp derivatives used in this study.

Fig. S2. Secreted Hbp707C/712C contains a disulphide bridge.

Fig. S3. Subcellular localization of secretion incompetent Hbp derivatives.

Supplementary Materials and Methods. Plasmid construction.

This material is available as part of the online article from <http://www.blackwell-synergy.com>